POLYKARYON FORMATION BY *MERCENARIA MERCENARIA* HEMOCYTES

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ABSTRACT

Examination of *Mercenaria mercenaria* hemocyte preparations on glass cover-slips showed that adherent, macrophage-like cells tended to form multinucleated giant cells morphologically similar to classical Langhans cells and foreign body inflammatory giant cells, as seen in vertebrates. The frequency of giant cell formation varied from clam to clam, possibly reflecting each animal's physiological state, as influenced by pollutant exposure, other environmental stressors and disease. Treatment of the hemocytes with concanavalin A promoted hemocyte aggregation and increased the rate of giant cell formation. Attempts to increase polykaryon formation by *in vivo* or *in vitro* phagocytic stimulation of the hemocytes were generally unsuccessful.

INTRODUCTION

Although the formation of polykaryons in response to inflammation has been frequently seen in vertebrates, little is known about the process in invertebrates. Giant cell formation in lower vertebrates was reported as early as 1926 by Lewis and Lewis in hanging-drop blood cell cultures. There are several reports of giant cells *in vivo* in mollusks; these cells were observed during postmortem examination of oysters by Sparks and Pauley (1964), and also associated with allografts and xenografts in gastropods by Cheng and Galloway (1970). These cells, designated "macrocytes," were thought to arise from fusion of mature granulocytes (Cheng, 1981). This study reports the *in vitro* formation of giant cells in hemocyte monolayer preparations from the bivalve *Mercenaria mercenaria*.

Multinucleated giant cells (MGC) can be classified as inflammatory foreign body cells (FBC), or Langhans cells (LC), based on morphological criteria (Chambers and Spector, 1982). Foreign body type polykaryons have numerous nuclei randomly distributed throughout their cytoplasm. In contrast, Langhans cells have nuclei arranged in circular or semi-circular patterns near the periphery of the cell. There appear to be many intermediate cell forms between the foreign body and Langhans types.

The exact manner by which giant cells arise has been the subject of controversy. It was proposed that karyokinesis in the absence of cytokinesis was responsible for the formation of the giant cell syncytium (Virchow, 1858). Borrel (1893) postulated that cell fusion was the primary phenomenon behind the formation of polykaryons. Current evidence favors the cell fusion theory (Mariano and Spector, 1974; Murch

et al., 1982). Observations made during the present study also tend to support the cell fusion model in *Mercenaria.*

MATERIALS AND METHODS

Commercially obtained *Mercenaria* were maintained at 8°–12°C in 30 gallon re-circulated artificial seawater aquaria.

A hypodermic needle was inserted into the posterior adductor muscle blood sinus to collect hemolymph samples. Hemolymph drops were placed on ethanol-cleaned coverslips, and the cells were allowed to adhere for 30–60 minutes in a moist chamber at room temperature. Nonadherent cells were gently washed off with sterile seawater (SSW) and the adherent cells bathed in medium. The medium consisted of 18 ml of cell-free (1500 × g, 15 min) hemolymph, 17.5 ml buffered seawater (pH 7, 0.05 M HEPES), 0.5 ml antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomy-cin), and 40 mg of glucose. This medium was sterilized by ultrafiltration (0.45 m pore size) and stored at 4°C. In long-term studies, the medium covering the hemocytes was changed daily. The use of buffered, antibiotic-containing medium was essential to prevent a subsequent overgrowth of contaminating bacteria. The cell monolayer preparations were rinsed with SSW, fixed in 10% formalin in SSW, washed, stained with hematoxylin, dehydrated by passage through an ethanol-water gradient, stained with eosin, cleared in xylene, and mounted on slides. Morphology was studied by brightfield and/or phase contrast microscopy.

Attempts were made to induce giant cell formation *in vitro* by adding various concentrations of foreign particulates to the medium. The particulates tested in-cluded yeast cells, latex beads, graphite, titanium dioxide, carbon, iron, aluminum, brass, or silica. Yeast cells, carbon, or the above-mentioned metal dust suspensions were also injected into the adductor muscle blood sinus in an attempt to induce poly-karyon production *in vivo.* Specific details of dosages and times for these studies will be given in the Results section. The effects of the addition to the medium of the plant lectin concanavalin A (Sigma Chemical Company) on giant cell formation by clam hemocytes were also determined.

RESULTS

Mercenaria hemocytes adhered rapidly and firmly to the glass substrate, and re-mained in an evenly dispersed cell monolayer for at least an hour (Fig. 1). Small cells containing irregular yellow-brown granules were seen in many of the cell preparations (Figs. 2, 3). The granules in these cells did not resemble the usual inclusions seen in *Mercenaria* granulocytes, or other hemocyte classes. Their relationship to the inclu-sions described in the so-called brown or serous excretory cells described by Ruddell and Wellings (1971) and Moore and Lowe (1977) remains to be ascertained.

Small MGC were present in some hemocyte preparations as soon as one hour in culture. These cells usually resembled small inflammatory giant cells; Figure 4 shows such a cell having five central nuclei from a 3 h culture. The nuclei in FBC usually appeared slightly swollen with light-staining, diffuse chromatin. The FBC continued to increase in size, until they contained numerous, randomly oriented nuclei (Fig. 5). Irregular yellowish granules, similar to those noted in small cells (Figs. 2, 3) prior to MGC formation, could be observed in the region of the nuclei of many FBC.

After about 6–8 h of maintenance, some of the hemocyte cultures contained small Langhans cells with characteristic ring-like arrangements of 8–20 nuclei (Fig. 6). The LC continued to develop for 24–48 h until they contained 50 to > 100 nuclei arranged in concentric circles near the periphery of the cell (Fig. 7).
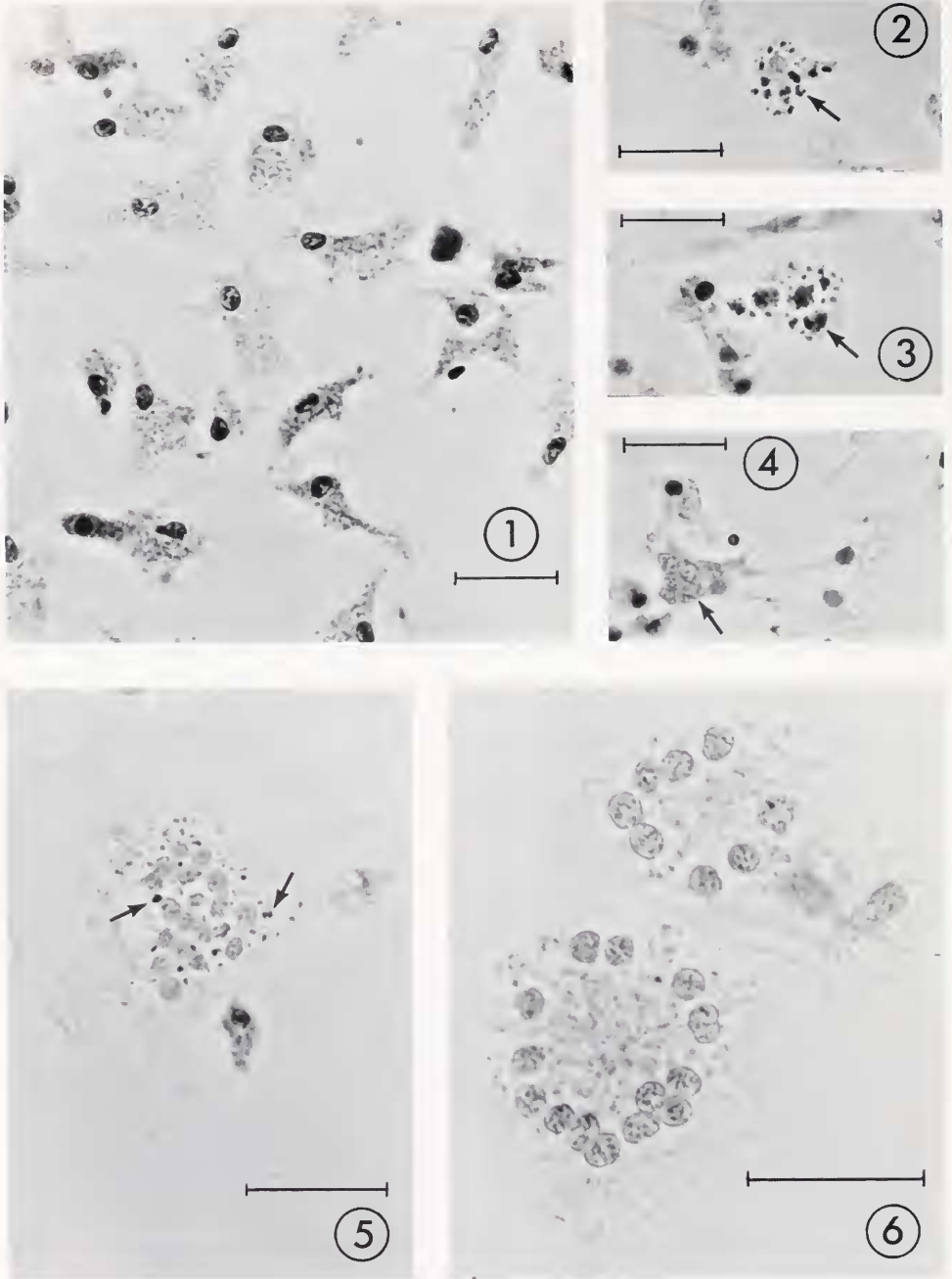
FIGURE 1.   Typical appearance of hemocyte monolayer 1 h after adhesion to the glass substrate.
FIGURE 2.   A hemocyte containing numerous irregular yellow granules (arrow).
FIGURE 3.   Hemocyte with large (2×5 μm) clumped yellow-brown inclusions (arrow).
FIGURE 4.   Early inflammatory foreign-body type cell after 3 h culture, arrow indicates one of five closely packed nuclei.
FIGURE 5.   Typical FBC after 5 h in culture containing 18 nuclei; one of many yellow-brown granules, similar to those in Figures 2 and 3, is indicated by the arrow.
FIGURE 6.   Langhans type cells in a 6 h hemocyte culture. Scale bar = 20 μm for Figures 1–6.

The FBC also continued to enlarge during 24–48 h in culture. In these prepara-
tions, FBC are often connected by numerous cytoplasmic strands (Fig. 8). The more
mature FBC frequently contained foreign material such as amorphous masses (Fig.
8), yellow-brown particles (Fig. 9) or cell-like structures (Fig. 10). The cell-like inclu-
sions were typically surrounded by a conspicuous vacuole; however, the yellow gran-
ules and amorphous material were not usually enclosed by vacuoles. Multinucleated
giant cells of either kind were rarely seen in >48 h hemocyte monolayer cultures;
possibly they lysed or became less adherent, and were lost during medium changes.

Giant cell formation was a highly variable phenomenon in *Mercenaria*. Groups
of 10–24 clams were examined at a given time; usually 50–60% of the individual
organisms per batch would yield MGC-producing hemolymph samples, but this
number could be as low as 5–10% in other batches. The actual number of cells in-
volved in giant cell formation represented a small portion of the total hemocytes
present in any particular monolayer preparation. Whereas a giant cell might contain
>50 nuclei, it was unusual to find more than 5–10 giant cells per coverslip, and each
preparation consisted of many thousands of hemocytes. Polykaryon formation was
followed in 20 batches of clams collected at roughly monthly intervals, but its fre-
quency did not correlate with any obvious parameters such as water temperature,
photoperiod or size of organism. The clams were obtained from commercial sources,
so nothing was known of their prior history, exposure to environmental stressors, etc.

Attempts were made to augment giant cell formation *in vitro,* by the addition of
foreign particles to the media overlying the hemocyte cultures, or *in vivo,* by injecting
the particles directly into the adductor muscle hemolymph sinus, and taking hemo-
lymph samples at intervals thereafter. The results of these studies will not be pre-
sented in detail because it was impossible to correlate statistically the frequency of
giant cell formation with either of the treatments. Metal particles were phagocytosed
in vitro to some extent, for example, carbon, graphite, aluminum, brass, or iron parti-
cles immediately adhered to the hemocytes and were ingested by 2–4 h. Silica and
titanium dioxide were infrequently actually phagocytosed, but remained bound to
hemocyte membranes. Considerable uptake of 2.02 μm diameter latex beads was
seen, but phagocytosis of 1.10 μm diameter beads was minimal. Both FBC and LC
were seen in many of the above-mentioned cultures, but at about the same frequency
as in the untreated control preparations. Yeast particles were rapidly ingested *in vitro*
and enclosed in large phagocytic vacuoles. In about half of the studies, phagocytic
stimulation of the hemocytes by yeast cell exposure caused a marked increase in giant
cell formation; however, repeated attempts to reproduce this phenomenon gave in-
consistent results. The rate of giant cell formation *in vivo* by hemocytes taken from
clams injected 1–48 h previously with particulates was comparable to that seen in
controls. Hemocytes from these animals often had cell surface-bound and/or ingested
particles. In some cases, *e.g.* 6 h after injection of 50 μg graphite in 0.5 ml SSW, the
foreign material was recovered in clumps enveloped by cellular capsules.

If con A was present in the medium, the hemocytes underwent membrane alter-
ations apparently leading to clumping and eventual fusion. The course of events fol-
lowing exposure to any given con A concentration was essentially the same, regardless
of length of exposure (2–48 h). The typical appearance of an early (6 h) untreated
hemocyte preparation is shown in Figure 11; the cells are rather evenly distributed
over the glass substrate. However, if the cells from the same animal were exposed to
Con A in the medium, the hemocytes showed a marked tendency to clump and form
syncytial masses (Fig. 12).

Exposure of hemocytes to 60–250 μg/ml con A produced numerous FBC and LC,
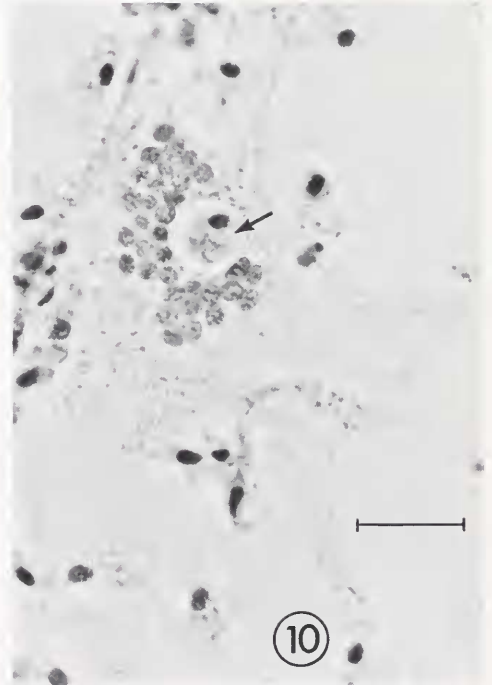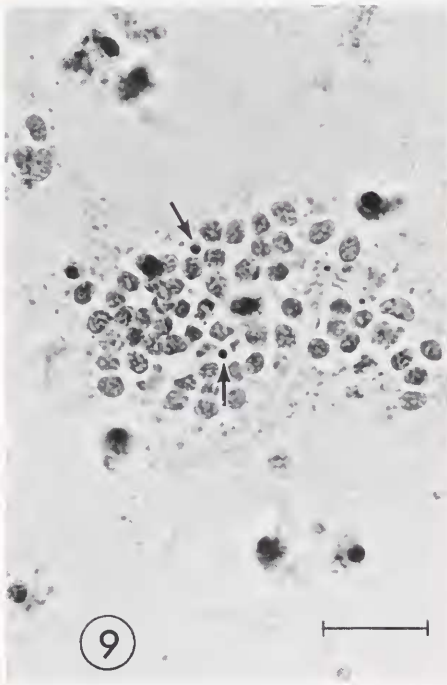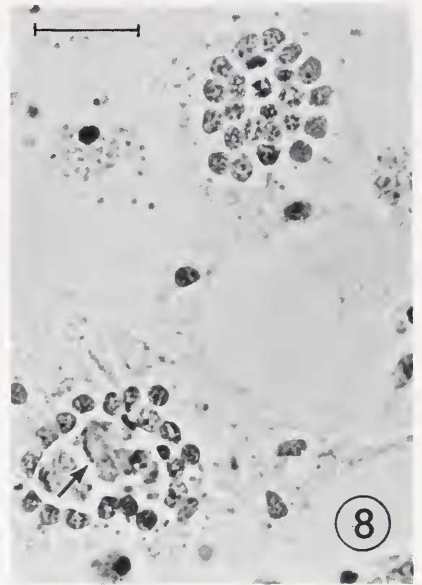as well as cell aggregations, in the hemocyte preparations from >80% of the clams
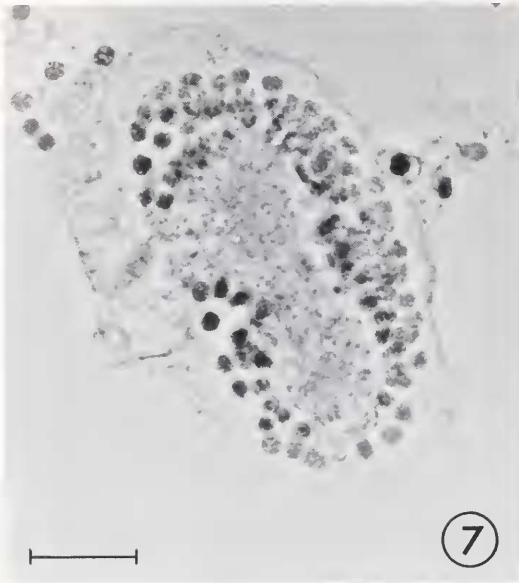
FIGURE 7. A mature LC in a 48 h preparation containing at least 80 nuclei.
FIGURE 8. Two FBC (24 h) connected by cytoplasmic strands, the lower cell contains unidentified amorphous material (arrow).
FIGURE 9. A mature FBC (48 h) with randomly arranged nuclei and several yellow-brown inclusions (arrows).
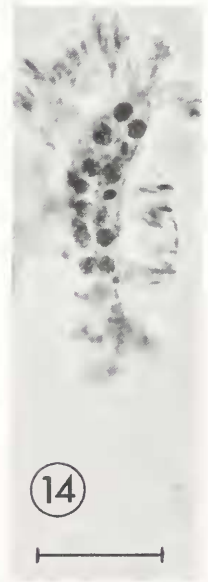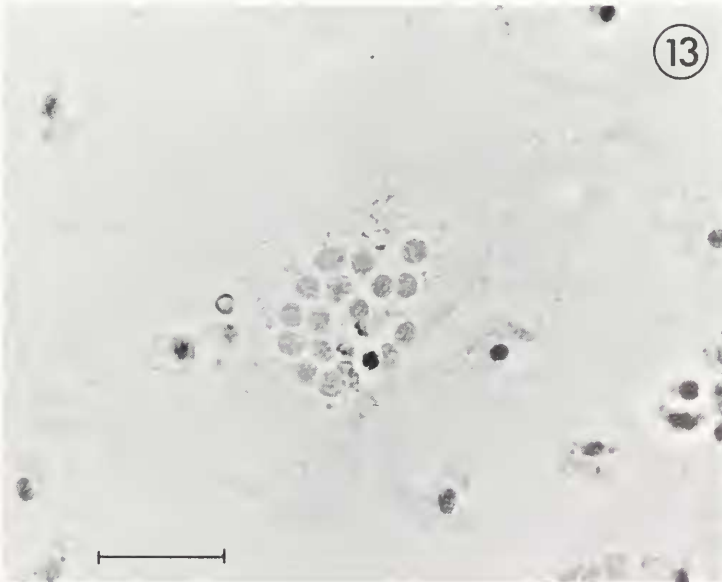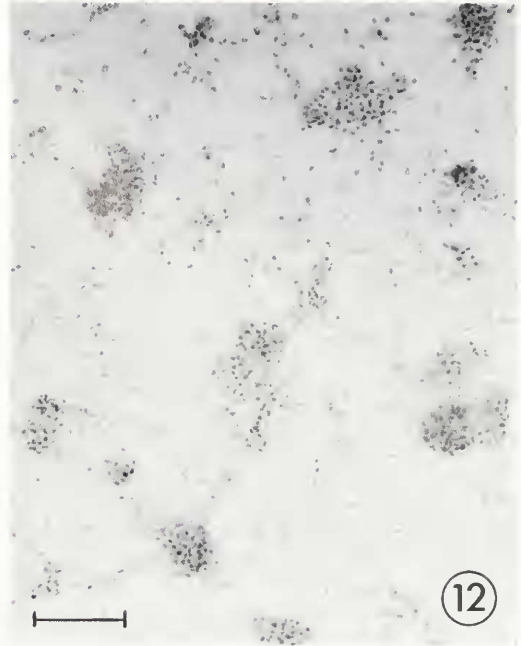FIGURE 10. A FBC (24 h) containing cell-like structure (arrow) in a large vacuole. Scale bar = 20 μm for Figures 7–10.

FIGURE 11.   Untreated adherent hemocytes after 6 h; cells are randomly dispersed over the prepara-
tion. Scale bar = 200 μm.
    FIGURE 12.   Cells from sample hemolymph sample as in Figure 11. Adherent cells were exposed to
250 μg/ml con A for 1 h and maintained for 5 h in the absence of con A. Many foci of aggregation are
present throughout the preparation. Scale bar = 200 μm.
    FIGURE 13.   Typical FBC in preparation exposed to con A (62.5 μg/ml, 24 h).
    FIGURE 14.   A FBC with highly ruffled membranes in a 24 h culture exposed to 125 μg/ml con A.
Scale bar = 20 μm for Figures 13 and 14.

studied. Cellular adhesion to glass was increased by exposure to con A, as shown by the unusually dense cell pellets remaining after vigorous washing with SSW. The morphology of MGC produced in the presence of con A was generally similar to that in spontaneously produced polykaryons (Fig. 13); however, occasionally FBC with highly ruffled membranes were present (Fig. 14). At higher con A concentrations (>250 $\mu g/ml$), hemocyte clumping and disruption were pronounced. The cells appeared to be extensively lysed, and the preparations contained filamentous networks and flocculent masses containing entrapped nuclei and debris.

Careful examination of 500 hemocytes in each of 10 monolayer preparations showed no evidence of mitotic activity; similarly, no sign of nuclear division was seen in any of 20 randomly selected giant cells. The apparent mitotic figure in Figure 13 is an artifact which is not seen in other focal planes of this cell. Since mitosis is very rare in hemocytes, either immediately after withdrawal or during our *in vitro* studies, the role of endomitosis in *Mercenaria* giant cell formation is probably minimal.

DISCUSSION

It is now accepted that MGC in laboratory rodents and other vertebrates arise from fusion of mononuclear phagocytes, based on direct observation (Sutton and Weiss, 1966; Bayliss, 1976), incorporation of labeled macrophage nuclei into MGC (Mariano and Spector, 1974), and the production of hybrid enzymes in MGC exudates from chimeric mice composed of two strains, each homozygous for one of the two isoenzymic forms of glucose-6-phosphate isomerase (Murch *et al.,* 1982). The blood phagocytes of most invertebrates examined so far show a number of morphological and biochemical similarities to the mononuclear leukocytes of vertebrates (Anderson *et al.,* 1973; Anderson, 1975; 1977; 1981). The ability of *Mercenaria* hemocytes to form several classical types of MGC is taken as another example of the early phylogenetic origins of macrophage-like cells. Multinucleated giant cells seen in these clam hemocyte cultures were virtually identical to those described in vertebrates. Several lines of indirect evidence point to fusion as the means of MGC formation in *Mercenaria*. Mitotic figures were almost never seen in individual hemocytes or in polykaryons. Polykaryon formation was rare at low cell densities, when the individual hemocytes were not in contact with each other. Concanavalin A was shown to promote hemocyte aggregation and MGC formation.

It is not clear that FBC and LC are distinct cell types, many intermediate forms exist in both *Mercenaria* and in vertebrates. Early ideas that FBC were formed only in response to the presence of insoluble foreign substances and that LC were restricted to granulomata associated with certain infections have proven to be subject to numerous exceptions. Most evidence supports the view that LC and FBC are actually different states of the same cell (Roizman, 1962). While there are reports that LC are the precursors of FBC (van der Rhee *et al.,* 1978; 1979), it is generally thought that LC arise from FBC, following microtubule-dependent reordering of the nuclei and other organelles (Mariano and Spector, 1974; Adams, 1976; Chambers and Spector, 1982). The results of this study suggest that a similar transformation of FBC to LC may occur in *Mercenaria,* because LC usually appeared in culture later than FBC, and because the LC:FBC ratio generally increased between 12–48 h. Long-term study of MGC in *Mercenaria* was difficult because the cells apparently became nonadherent or lysed after several days in culture. Murine MGC were also reported to have a short lifetime (Papadimitriou and Sector, 1971), with the actual life span inversely proportional to the number of nuclei per cell (Papadimitriou and Walters, 1979).

The exact stimulus for MGC formation by *Mercenaria* hemocytes is unknown,

but several suggestions have been proposed to account for macrophage fusion in higher animals. There are reports that fusion is directed by lymphokines released after antigenic stimulation (Galindo, 1972; Parks and Weiser, 1975), but many of the agents most commonly used to induce MGC formation are poorly antigenic. Indeed, thymectomy was shown to have little effect on MGC production (Papadimitriou, 1976). In this study, it is unlikely that soluble blood cell products, either secreted into the hemolymph or released from accidental hemocyte lysis during hemolymph collection and subsequent handling, mediated MGC formation. All hemocyte preparations were maintained in media containing pooled, cell-free hemolymph, which presumably would contain hemocyte products, but MGC formation was seen in only certain samples. The evidence strongly suggests that the capability to spontaneously form MGC was restricted to individual *Mercenaria,* and was not merely a result of *in vitro* culture of hemocytes. It was suggested that MGC were produced as a result of recognition of cell surface abnormalities on ageing or otherwise altered macrophages (Mariano and Spector, 1974), but various experimental alterations of macrophage surfaces failed to lead to the formation of polykaryons (Chambers, 1977a). Another possible theory (Chambers, 1977b) suggests that MGC form after the simultaneous attachment of several macrophages to the same ingestible material. The cells then fuse in the course of their efforts to ingest the same particle; cell membrane fusion normally accompanies the phagocytic process.

Attempts were made to promote MGC formation by *Mercenaria* hemocytes *in vivo* and *in vitro* by exposure to a variety of foreign particles. Whereas many kinds of particles were phagocytosed, in few cases were there clear increases in MGC formation caused by competition by the cells for the material in question, or as an incidental consequence of the cells ingesting material in close proximity to other hemocytes, as suggested by Chambers and Spector (1982).

The plant lectin con A promoted MGC formation in *Mercenaria* hemocyte cultures; both FBC and LC were more abundant after con A treatment. Berman and Stulberg (1962), Ptak *et al.* (1970), Smith and Goldman (1971), and Chambers (1977c, d) using lectins or antimacrophage serum, showed that if human or rodent macrophages are brought into contact before ingestion of surface-bound material, subsequent incubation will result in cell fusion. These molecules which induce MGC formation after interaction with the membrane may mimic the action of natural moieties generated in the inflammatory environment, and may account for the apparent lack of foreign material in certain MGC when examined under the microscope.

Irregular, yellow-brown granules, resembling those of brown or serous cells, were seen free in the cytoplasm of FBC, or still contained in apparently intact cells within the giant cells. It is interesting that cell-like inclusions can also be found in murine MGC (see Fig. 5 in Mariano and Spector, 1974). The central location of this material in clam FBC of all sizes suggests a possible role in MGC initiation, possibly following phagocytosis. The presence of foreign material in the cytoplasm suggested to early workers that augmented phagocytosis was a major function of MGC. However, more recent work showed that giant cells have a lower phagocytic potential than an equivalent mononuclear cell population. In fact, it was shown that both reduction in phagocytic activity and loss of cell surface receptors accompany the development of MGC (Papadimitriou *et al.,* 1975; Chambers, 1977e).

The granulomatous inflammatory response in vertebrate animals has received continuous scientific study for many years. Comparable information on invertebrates is not as comprehensive. Mammalian granulomas can be defined as a compact, organized collections of mature mononuclear phagocytes (Adams, 1976); frequently other elements of the mononuclear system are also present, including epithelioid cells

and multinucleated giant cells. Giant cells are commonly associated with inflammatory responses to bacterial and viral infections, implanted foreign material, and/or neoplasms. Preliminary evidence presented here suggests that giant cells are also found in invertebrates, and that they are probably derived, as is the case in higher animals, from fusion of phagocytic leukocytes.

ACKNOWLEDGMENTS

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